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The Breast Cancer Susceptibility gene 1 (BRCA1) encodes an 1863-amino acid protein that plays a central role in the pathogenesis of hereditary breast cancer. The BRCA1 protein contains an N-terminal RING finger and two C-terminal BRCT domains (BRCT1 and BRCT2), which are critical for BRCA1-mediated tumor suppression and are targets for cancer-causing mutations. The BRCA1 RING interacts with the RING domain of BARD1, another protein involved in breast cancer pathogenesis, and with the C-terminal domain of BAP1 (amino acids 598-729), a ubiquitin hydrolase that enhances BRCA1-mediated cell growth suppression, whereas the BRCA1 BRCT domains interact with the C-terminal region of BACH1 (amino acids 888-1063), a helicase-like protein that contributes to the BRCA1 DNA repair function. The present project focuses on the elucidation of the structural basis of the BRCA1 RING and BRCT domain interaction with the proteins BARD1, BAP1, and BACH1 using X-ray crystallography. In the second year of the award we completed the Tasks 2a, 2b, and 2c of the Statement of Work. For the remaining year of the award, we propose to determine the crystal structures of the BRCA1(residues 1-103)/BAP1(residues 598-729), BRCA1(residues 1-304)/BARD1(residues 25-189), and BRCA1(1650-1863)–BACH1(984-995) phosphopeptide complexes.

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INTRODUCTION

The Breast Cancer Susceptibility gene 1 (BRCA1) encodes an 1863-amino acid protein that plays a central role in the pathogenesis of hereditary breast cancer (1-3). The BRCA1 protein contains an N-terminal RING finger and two C-terminal BRCT domains (BRCT1 and BRCT2), which are critical for BRCA1-mediated tumor suppression and are targets for cancer-causing mutations (1-4). The BRCA1 RING interacts with the RING domain of BARD1, another protein involved in breast cancer pathogenesis, and with the C-terminal domain of BAP1 (amino acids 598-729), a ubiquitin hydrolase that enhances BRCA1-mediated cell growth suppression, whereas the BRCA1 BRCT domains interact with the C-terminal region of BACH1 (amino acids 888-1063), a helicase-like protein that contributes to the BRCA1 DNA repair function (4-7). Importantly, it has been shown recently that the BRCA1 BRCT domains interact with phosphoserine- or phosphothreonine-containing peptides, including the BACH1 peptide ISRTST(pS)PTFNK, where pS denotes phosphorylated Ser990 (8-10). Moreover, a heterodimer consisting of the RING-encompassing regions of BRCA1 (amino acids 1-304) and BARD1 (amino acids 25-189) functions as a ubiquitin ligase targeting cellular proteins for destruction, whereas the individual BRCA1 and BARD1 domains have very low ubiquitin ligase activities (5). This project focuses on the elucidation of the structural basis of the BRCA1 RING and BRCT domains interaction with BARD1, BAP1, and BACH1, using X-ray crystallography. Because BRCA1 plays a central role in breast cancer pathogenesis, the determination of its three-dimensional structure will facilitate the unraveling of the molecular mechanisms of its function in breast carcinogenesis. The Specific Aims of this proposal are:

Specific Aim 1. To co-crystallize and determine the crystal structure of the BRCA1 RING (amino acids 1-103) bound to the C-terminal domain of BAP1 (amino acids 598-729).

Specific Aim 2. To determine the crystal structure of the ubiquitin ligase heterodimer consisting of the BRCA1 region spanning amino acids 1-304 and the BARD1 region spanning residues 25-189.

Specific Aim 3. To co-crystallize and determine the crystal structure of the BRCA1 BRCT1/BRCT2-containing region (amino acids 1650-1863) complexed with the C-terminal region of BACH1 (amino acids 888-1063).

BODY

During the second year of the award we proceeded with the completion of the experiments proposed in Task 2 of the revised Statement of Work (as described in the first year's progress report). A description of our progress in these studies follows below.

Task 2a. To clone the BRCA1 BRCT1/BRCT2-containing region (residues 1650-1863), the BAP1 C-terminal domain (residues 598-729), and the BACH1 region (residues 888-1063) into prokaryotic expression vectors for production in *E. coli* cells (months 13-16). The DNA fragment encoding the human BRCA1 BRCT1/BRCT2 domain (amino acids 1650-1863) was amplified with the polymerase chain reaction (PCR) using the human BRCA1 cDNA as a template and cloned into a modified pTYB12 prokaryotic vector (New England BioLabs), following standard protocols (11). The resulting construct was verified by DNA sequencing and was used to transform *E. coli* ER2566 cells (NEB).

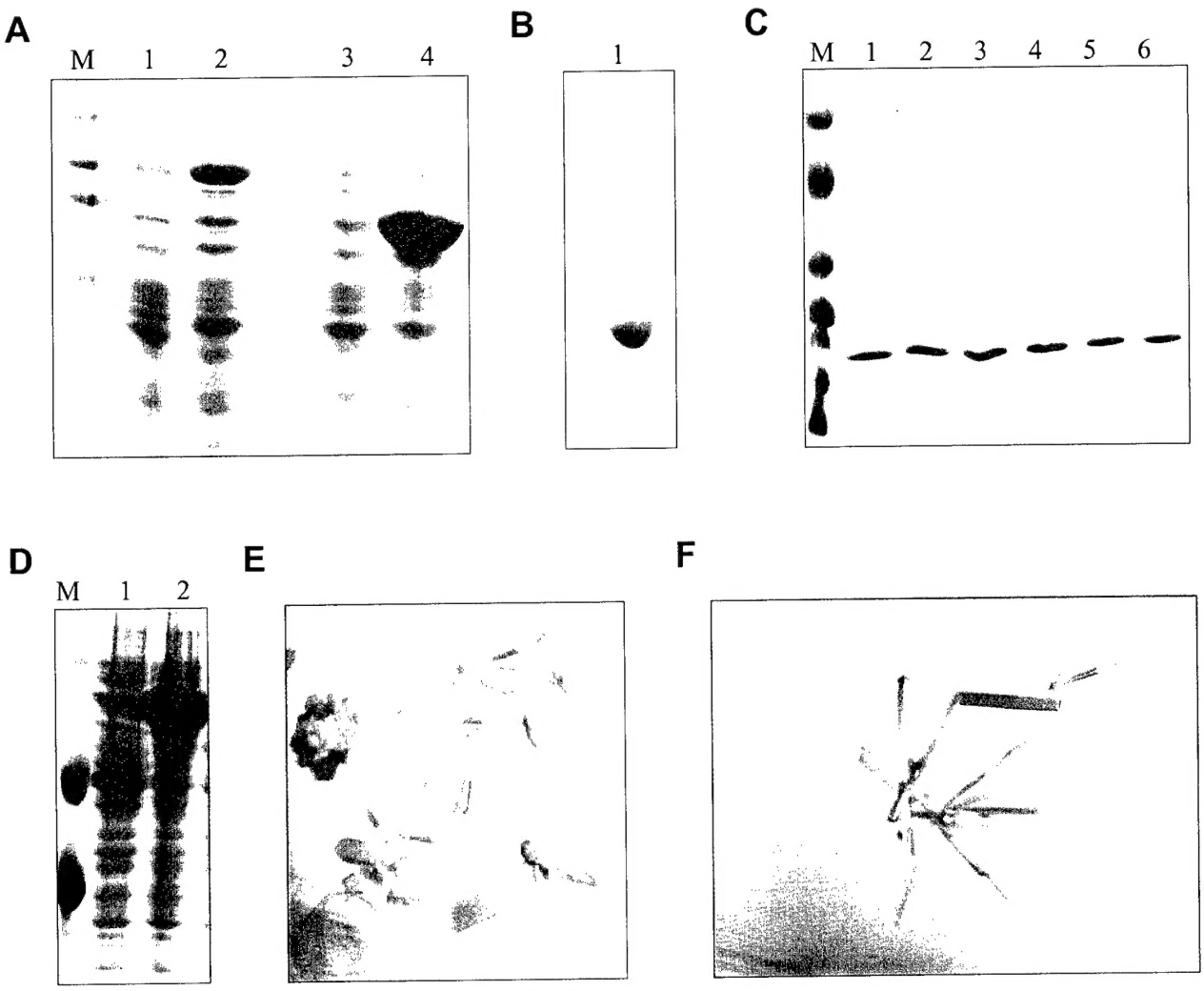

Figure 1. (A) Expression of the BRCA1 BRCT1/BRCT2 domain (amino acids 1650-1863) and the BAP1 C-terminal domain (amino acids 598-729). SDS-PAGE of intein-BRCT1/BRCT2 and intein-BAP1 fusion proteins produced in *E. coli* cells. *Lane M*: protein markers; *lanes 1 and 2*: uninduced and induced whole-cell extracts, respectively, of *E. coli* ER2566 cells expressing intein-BRCT1/BRCT2 protein; *lanes 3 and 4*: uninduced and induced whole-cell extracts, respectively, of *E. coli* cells expressing intein-BAP1(598-729) protein. **(B)** Purified BRCT1/BRCT2(1650-1863) protein following overnight cleavage from intein with DTT at 4°C and concentration. **(C)** *Lane M*: protein markers; *lanes 1-6*: eluted fractions of purified BAP1(598-729) protein following overnight cleavage from intein with DTT at 4°C. **(D)** Expression of the BACH1 domain (residues 888-1063). SDS-PAGE of intein-BACH1 fusion protein produced in *E. coli* ER2566 cells. *Lane M*: protein markers; *lanes 1 and 2*: uninduced and induced whole-cell extracts, respectively, of *E. coli* cells expressing intein-BACH1 protein. **(E)** Microcrystalline plates of recombinant BRCA1 RING protein (residues 1-103) bound to BAP1 C-terminal domain (amino acids 598-729), grown using the sitting drop vapor diffusion method. **(F)** Needle-like crystals of the BRCA1 BRCT1/BRCT2 domain (amino acids 1650-1863) bound to the BACH1 phosphoserine-containing peptide ISRTST(pS)PTFNK, grown using the sitting drop vapor diffusion method.

To clone the C-terminal domain of human BAP1 we obtained the cDNA KIAA0272 from Dr. Takahiro Nagase at the Kazusa DNA Research Institute, Japan, which codes for the human BAP1 protein (accession number D87462). Using this cDNA as a template, we amplified by PCR a DNA fragment encoding the BAP1 C-terminal domain (residues 598-729) and cloned it into a modified pTYB12 prokaryotic vector, using standard methods. The resulting construct was verified by DNA sequencing and was used to transform *E. coli* ER2566 cells (NEB).

Several requests to obtain the human BACH1 cDNA from other laboratories were denied, so we proceeded with cloning the DNA fragment coding for the BACH1 region (residues 888-1063) from a human brain cDNA library (Clontech) using PCR. The obtained DNA fragment was cloned into a modified pTYB12 prokaryotic vector. The resulting construct was verified by DNA sequencing and was used to transform *E. coli* ER2566 cells (NEB).

Task 2b. To express the BRCA1 BRCT1/BRCT2-containing region (residues 1650-1863), the BAP1 C-terminal domain (residues 598-729), and the BACH1 region (residues 888-1063) in *E. coli* cells (months 17-24). The BRCA1 BRCT1/BRCT2 protein was produced in ER2566 cells as a fusion with intein. Expression of the fusion protein was induced at 20°C by 1 mM IPTG at OD_{600} of 0.6 (Figure 1A, lanes 1,2), purified on chitin beads (NEB) using the manufacturer's protocols, was further purified by size-exclusion chromatography and was concentrated using Centriprep concentrators (Amicon) for use in crystallization experiments (Figure 1B), as we described previously (12-17).

For the production of the BAP1(598-729) recombinant protein, we followed a similar approach. Briefly, expression of the BAP1(598-729) protein as a fusion with intein in ER2566 cells was induced at 20°C by 1 mM IPTG at OD_{600} of 0.6 (Figure 1A, lanes 3,4), purified on chitin beads (NEB) using the manufacturer's protocols (Figure 1C), was further purified by size-exclusion chromatography and was concentrated using Centriprep concentrators for use in crystallization experiments, as we described previously (12-17).

The recombinant protein BACH1(888-1063) was produced in ER2566 cells as a fusion with intein following a similar strategy. Expression of the fusion protein was induced at 22°C by 1 mM IPTG at OD_{600} of 0.6 (Figure 1D, lanes 1,2), purified on chitin beads (NEB) using the manufacturer's protocols, was further purified by gel filtration and was concentrated using Centriprep concentrators for use in crystallization trials, as we described previously (12-17). In general, using this expression system we were able to obtain up to 5-7 mg of recombinant protein (>98% pure as estimated by SDS-PAGE) per liter of bacterial culture.

Task 2c. To crystallize the BRCA1 BRCT1/BRCT2(1650-1863) in complex with BACH1(888-1063) and the BRCA1(1-103) in c omplex with BAP1(598-729), using sparse matrix vapor diffusion crystallization methods, seeding, limited proteolysis, and mutagenesis to improve crystallization (months 18-24). Having purified these proteins to near homogeneity, we proceeded with crystallization experiments. The protein complexes BRCA1(1650-1863)–BACH1(888-1063), BRCA1(1-103)–BAP1(598-729), and BRCA1(1650-1863)–BACH1(984-995) phosphopeptide, were formed by mixing the corresponding proteins at stoichiometric ratios, followed by purification of the complexed proteins by gel filtration. Subsequently, the protein complexes were concentrated and used for crystallization experiments, as described in the original application. To date, the protein complex BRCA1(1650-1863)–BACH1(888-1063) did not yied any crystals. By contrast, small crystals were obtained with the BRCA1(1-103)–BAP1(598-729) complex (Figure 1E), and the BRCA1(1650-1863)–

BACH1(984-995) phosphopeptide complex (Figure 1F). Currently we are in the process of optimizing the crystallization conditions in order to obtain large single crystals of these complexes that diffract to high resolution and determine the structures of these proteins.

KEY RESEARCH ACCOMPLISHMENTS

1. Cloning of the BRCA1 BRCT1/BRCT2-containing region (residues 1650-1863) into a modified pTYB12 prokaryotic expression vector for production in *E. coli* cells (*Task 2a*).
2. Cloning of the BAP1 C-terminal domain (residues 598-729) into a modified pTYB12 prokaryotic expression vector for production in *E. coli* cells (*Task 2a*).
3. Cloning of the BACH1 region (residues 888-1063) into a modified pTYB12 prokaryotic expression vector for production in *E. coli* cells (*Task 2a*).
4. Expression of the BRCA1 BRCT1/BRCT2 (residues 1650-1863) in *E. coli* ER2566 cells and purification of the recombinant protein using chromatographic methods (*Task 2b*).
5. Expression of the BAP1 C-terminal domain (residues 598-729) in *E. coli* ER2566 cells and purification of the recombinant protein using chromatographic methods (*Task 2b*).
6. Expression of the BACH1 region (residues 888-1063) in *E. coli* ER2566 cells and purification of the recombinant protein using chromatographic methods (*Task 2b*).
7. Crystallization experiments of the BRCA1 BRCT1/BRCT2(1650-1863) protein bound to BACH1(888-1063) protein using sparse matrix vapor diffusion crystallization methods, so far producing no crystals (*Task 2c*).
8. Crystallization experiments of the BRCA1 BRCT1/BRCT2(1650-1863) protein bound to the BACH1(985-995) phosphopeptide ISRST(pS)PTFNK using sparse matrix vapor diffusion crystallization methods, resulting in small crystalline plates (*Task 2c*).
9. Crystallization experiments of the BRCA1 RING(1-103) protein bound to BAP1 C-terminal domain (residues 598-729) using sparse matrix vapor diffusion crystallization methods, resulting in small needle-like crystals (*Task 2c*).

REPORTABLE OUTCOMES

The experiments of the second year are still in progress and no new publications have resulted so far.

CONCLUSIONS

In the second year of the award we completed the Tasks 2a, 2b, and 2c of the revised Statement of Work. In addition, the recent discovery that the BRCA1 BRCT1/BRCT2 domains interact with phosphoserine- or phosphothreonine-containing peptides, including the BACH1 peptide ISRTST(pS)PTFNK (8-10), provided a compelling reason for us to initiate crystallization experiments of these domains complexed with this peptide, a task that was not included in the original Statement of Work. The crystal structure of BRCA1 BRCT1/BRCT2 domains bound to the target phosphopeptides will provide much more information than the structures of these domains in the unbound form (18). The information obtained from these

experiments will elucidate the mechanisms underlying regulation of BRCA1 by BAP1 and BACH1 and will reveal the molecular changes induced by cancer-causing mutations in the BRCA1 domains that affect their interaction with BAP1 and BACH1.

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